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(71) Applicant *(for all designated States except US)*: HOECHST MARION ROUSSEL LTD. [JP/JP]; 17–51, Akasaka 2–chome, Minato–ku, Tokyo 107–8465 (JP).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: KAWAI, Shinji [JP/FR]; 416, rue Emile Dubois, F–75014 Paris (FR). SUGIURA, Takeyuki [JP/JP]; 4–4–22–202, Kamishakuji–i, Nerima–ku, Tokyo 177–0044 (JP). HÖTTEN, Gertrud [DE/DE]; Hölkeskampring 96, D–44625 Herne (DE).

(74) Agent: VIEILLEFOSSE, Jean–Claude; Hoechst Marion Roussel, 102, route de Noisy, F–93235 Romainville Cedex (FR).

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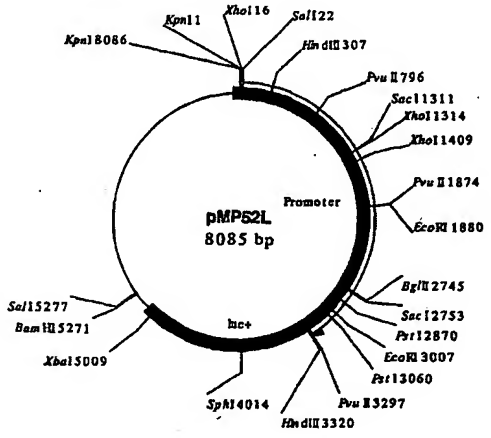
(54) Title: HUMAN MP52 GENE PROMOTER AND METHOD FOR EXPLORING USEFUL SUBSTANCE BY USING THE SAME



(57) Abstract

The present object is to provide a method for exploring low molecular weight compounds having osteogenetic activity or an activity to inhibit osteogenesis that shows an equal degree to human BMP proteins. A method for exploring low molecular weight compounds which regulate positively or negatively the expression of human MP52 with reference to a reporter acivity by using 5' upstream region gene containing the human MP52 promoter and an animal cell into which a recombinant expression vector that has been ligated to an appropriate reporter gene has been introduced. Low molecular weight compounds and their derivatives obtained by the present method have morphogenetic activity and inhibiting activity for bone and cartilage, inducing or inhibiting activity for tendon and ligament, forming, inducing, and protecting effects on neural cells, or inducing or inhibiting angiogenic activity, and are effective as preventive or therapeutic agent for cartilage and bone diseases, therapeutic agent for injury of tendon and ligament, therapeutic agent for neural diseases, or healing agent for injury and carcinostatic, through the expression of human MP52.

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HUMAN MP52 GENE PROMOTER AND METHOD FOR EXPLORING USEFUL SUBSTANCE BY USING THE SAME

BACKGROUND OF THE INVENTION

(1) Field of the Invention

The present invention relates to a 5' upstream region DNA sequence which contains a promoter of human MP52 gene. Furthermore, the present invention provides a method for exploring low molecular weight compound regulating positively or negatively the expression of human MP52 by using a mass of animal cells transfected with a recombinant expression vector harboring the 5' upstream region DNA containing the human MP52 gene promoter in front of a suitable reporter gene, and by using a reporter activity as an indicator.

(2) Description of the Related Art

Many substances have been known as usable for healing of bone deficit or fracture, by enhancing bone regeneration. Among them, a bone morphogenetic protein (BMP, hereafter) is a protein belonging to TGF (transforming growth factor) -β superfamily and has been formerly found as a substance inducing ectopic ossification and existing in decalcified bone tissue (Trends in Biotechnol. 11, 379-383, 1993). BMP members known so far range from BMP-1 to BMP-14. Among them, the members from BMP-2 to BMP-14 have been known as showing the bone morphogenetic activity.

Human MP52 is a BMP-like protein isolated by PCR cloning (Biochem. Biophys. Res. Comm. 204, 646-652, 1994). Recombinant human MP52 has been reported as effective not only in inducing ectopic ossification, but also in curing bone deficit in an animal model (Growth Factors 13, 65-74, 1996).

It is considered that these BMP proteins are effective in treating and preventing various bone disorders and bone diseases. However, the BMP proteins exist in very small quantity in nature, and for an available large quantity for therapeutic use, manufacturing a recombinant protein is necessary.

Preparing a recombinant protein is generally very

expensive compared with low molecular weight compounds. Furthermore, there are many restrictions as a medicinal drug in terms of physical properties and administration methods due to proteineous characteristics. Considering these points, a small molecular organic compound having the activity equal to that of the BMP proteins described above, if any, should be a highly promising medicinal drug.

SUMMARY OF THE INVENTION

The present invention provides a method for exploring a substance which induces human MP52, a BMP-like protein. A useful substance obtained by this exploring method can be effective in inducing expression of human MP52, a bone formation factor. Since it has a small molecular weight and the same activity as that of human MP52, it can have a very useful application. On the contrary, the present invention provides a method for exploring a substance which inhibits expression of human MP52. In case that human MP52 relates to bone and cartilage hyperplasia, it can prevent hyperplasia by inhibiting the expression of human MP52.

Recently, it has been suggested that a mutant of the human MP52 protein not only decreases the activity per se, but also deactivates other BMPs by forming dimer proteins (Nature Genetics, 17, 58-64, 1997). The substance inhibiting the expression of human MP52 may be effective in mitigating chondrodystrophy caused by such a mutant MP52.

Moreover, it has been known that human MP52 has a tendon and ligament inducing activity (Toshiyuki Tashiro et al., American Society of Bone and Mineral Research, F336, 1997), neural cells forming, inducing, and protecting effects (J. Neurosci. Res., 42, 724-732, 1995), and angiogenic activity (Exp. Cell Res., 235, 218-226, 1997). Thus, it is possible that the exploring method provided by the present invention gives tendon and ligament inducing and inhibiting substances, neural inducing substances, or pro- and anti-angiogenic substances.

For such an exploring method, an example was so far only reported using a mouse BMP-2 promoter (W097/15308); there is no example of using the human MP52 promoter. In addition,

since the materials of the exploring method provided by the present invention are all derived from human sources, it can be expected that the discovered compounds should show the clinical effects.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a 3.5 kb of 5' upstream region of human MP52 gene and a restriction enzyme map.

Fig. 2 is a recombinant expression vector (pMP52L) containing a 5' upstream region of human MP52 gene. A fragment, base No. from 1 to 3227 (*Apa*1 position 1) shown in SEQ ID No.: 1 of the Sequence Listing, was inserted to the restriction enzyme site, *Hin*dIII-*Kpn*I, of pGL3-basic.

Fig. 3 is a result of measuring a human MP52 promoter activity (transient expression). pGL3B means pGL3-basic in the figure.

DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention relates to a DNA whose nucleotide sequence is represented by the base sequence from No. 1 to No. 3521 in SEQ ID NO.: 1 of the Sequence Listing that encodes a human MP52 gene promoter region, or a fragment thereof. SEQ ID NO.: 1 of the Sequence Listing shows a 5' upstream region sequence of the human MP52 gene.

The present invention relates to a method for preparing the DNA shown in SEQ ID NO.: 1 of the Sequence Listing having the steps of:

(1) preparation of a cDNA probe by PCR with a degenerate primer,

(2) plaque hybridization screening of a human genomic phage library by using the probe, and

(3) subcloning of a target fragment from an isolated phage clone.

The plasmid vector used here is not restricted and any commercialized vector can be used. A preferable example is pUC18.

The present invention relates to a recombinant expression vector characterized by integration of the full length or a part of DNA shown in SEQ ID NO.: 1 of the Sequence Listing into a reporter gene. In detail, the

recombinant expression vector is constructed to locate a suitable region of 5' upstream region of the human MP-52 gene, that is represented by SEQ ID No.: 1 of the Sequence Listing, in front of a reporter gene. The reporter gene such as luciferase or β-galactosidase gene shows an expressing status on behalf of an original product. The basic vector for constructing the recombination expression vector is not specially restricted to allow to use a plasmid vector commercialized. The present invention uses pGL3-basic as a preferable example. The use of pGL3-basic yielded a pMP52L (8085 bp) that is a recombination vector containing the human MP52 promoter and a luciferase reporter gene. The present invention assigned it to a recombination expression vector. It is necessary to introduce the vector, to mammalian cells, preferably a human osteoblast-like cells, such as SaOS-2 cells, with a liposome. The animal cells stably transfected with the recombinant expression vector are selected by using a resistance marker.

The present invention relates to an exploring method for a useful substance by using a recombinant expression vector characterized by integrating the full length or a part of DNA shown in SEQ ID NO.: 1 of the Sequence Listing into a reporter gene. The sequence from 2880 to 3521 in SEQ ID NO.: 1 of the Sequence Listing has been already reported (Biochem. Biophys. Res. Comm., 204, 646-652, 1994). The longer sequence has been integrated in a recombinant expression vector, the more natural process of the exploring method can be used. The present invention relates to the method for exploring a useful substance which is a bone-related substance. In particular, the present invention relates to the method for exploring a useful substance which is an osteogenesis inducing substance or which is an osteogenesis inhibiting substance. Furthermore, the present invention relates to the method for exploring a useful substance which is a neurogenic substance or which is an angiogenic substance. A low molecular weight compound which induces or inhibits the expression of human MP52 can be obtained by isolating the promoter which regulates the expression of the

gene, by ligating it to a suitable reporter gene and by introducing the gene structure into a suitable mammal cell to make an exploring system. The substance which regulates the expression of human MP52 in the exploring system works on the promoter to increase or decrease the expression level of the reporter gene. Therefore, a simple and easy measurement of the reporter activity makes an exploration of the aimed possible substance.

The animal cell transfected with said vector can be used for a method for screening a chemical compound library by high throughput screening (Nature, 384, supp, 14-16, 1996) and finding an active substance from natural compounds. The substance which increases or decreases an activity is searched by treating the cell with a compound for an appropriate period of time and measuring a reporter activity. The compound obtained in such a way can regulate the expression by working directly on a transcription factor or indirectly on the promoter of human MP52 through regulating a signal transduction system. Therefore, these compounds are effective as a therapeutic agent for osteocartilaginous diseases, cancer metastasis to bone, or osteohyperplasia. As other examples, these compounds are effective as a recovering agent for tendon and ligament injuries, a remedy for neural diseases, or an angiogenesis inducing or inhibiting agent.

The substance obtained by the present invention has a bone or cartilage morphogenetic activity and is effective as an agent for therapeutic and preventive treatment in the field of orthopedic surgery (fracture, osteoarthritis such as joint osteoarthritis and hip joint osteoarthritis, arthrosteitis, damage of cartilage such as damage of meniscus, regeneration of bone and cartilage deficit caused by injury and tumor dissection, bone reconstruction such as spinal fusion and vertebral canal enlargement, and congenital cartilage and bone diseases such as dysosteogenesis and achondroplasia), or dental fields (bone reconstruction such as palatoschisis, mandible reconstruction, and residual ridge construction), and osteoporosis. Moreover, the substance of the present invention can be used for bone graft in aesthetic

surgery. These therapeutic treatments are effective in treating the field of veterinary surgery. On the other hand, the present invention can provide a substance which inhibits bone or cartilage morphogenesis. In this case, the substance is applied as an agent to prevention and therapy of bone or cartilage hyperplasia.

Moreover, the substance which induces or inhibits tendon and ligament can be obtained according to the present invention. Therefore, the present invention provides a method for exploring medicinal drugs to treat damage of tendons and ligaments. Furthermore, since human MP52 has neural cells forming, inducing, and protecting effects, the compounds obtained from the present invention can be applied as therapeutic agents for neural diseases. In addition, it is possible to obtain a substance inducing or inhibiting angiogenesis. The compounds obtained can be used as remedy for injury, carcinostatic and metastasis inhibitor.

By using 5' upstream region containing the human MP52 gene promoter and an animal cell into which a recombinant expression vector that has been integrated in an appropriate reporter gene, low molecular weight compounds regulating positively or negatively the expression of human MP52 can be explored with reference to a reporter activity. Low molecular weight compounds and their derivatives have morphogenetic activity and inhibiting activity for bone and cartilage through the expression of human MP52 and are useful as preventive or therapeutic agents for cartilage and bone diseases, remedies for osteometastasis, or therapeutic and preventive agents for osteohyperplasia. Furthermore, these compounds are effective as repairing agent for injury of tendon and ligament, therapeutic agent for neural diseases, and inducing and inhibiting agent for angiogenesis.

EXAMPLES

This invention shall be more illustratively explained by way of the following Examples. The following Examples are to be considered in all respects as illustrative and not restrictive.

Example 1 : Cloning of 5' upstream region of human MP52 gene

The 40 cycles of PCR reaction was carried out by using the primer OD of SEQ ID No.: 2 of the Sequence Listing and the primer OID of SEQ ID No.: 3 of the Sequence Listing, cDNA derived from a human fetus (8-9 week old) as a template, and 1.5 unit Taq polymerase (provided from Perkin Elmer Cetus). The following processes were repeated twice: the DNA amplified was treated with *Sph*I and *Alw*NI and then the PCR reaction was carried out 13 times. Finally, it was treated with *Eco*RI, the PCR product was ligated to pBluescript SK (provided from Stratagene Ltd.). A human genomic phage library (provided from Stratagene Ltd.) was screened by plaque hybridization using the cDNA sequence of MP52 among cloned sequences. A 20 kb DNA obtained was subcloned to pBluescript SK vector, and an unnecessary part to remove was cleaved by restriction with *Sal*I and *Bam*HI, to obtain the pBluescript vector containing a human MP52 DNA of 8.8 kb. The vector was named E. coli pMP52. E. coli pMP52 was deposited in National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry 1-3, Higashi 1-chome, Tsukuba-shi Ibaraki-ken 305-8566 Japan, in March 30, 1998 with depository No. FERM P-16734 and transferred to the International Depository Authority under Budapest Treaty on April 1, 1999 (Deposit No. FERM BP-6688).

Example 2 : Determination of DNA sequence of 5' upstream region of human MP52 gene

The pBluescript vector containing a human MP52 DNA of 8.8 kb was digested by *Xba*I, a remained large fragment (7.6 kb) was purified by agarose electrophoresis to subject to self-ligation by using a ligation kit provided from Takara Shuzo Ltd. The 3.5 kb sequence of the 5' terminal was determined by using a vector containing the upstream region of the human MP52 gene of 4.7 kb as a template and by a ALF automated DNA sequencer (proviced from Amersham Pharmacia Biotech Ltd.)(Proc. Natl. Acad. Sci. USA 74, 5463-5467, 1977). The 5' upstream region of the human MP52 gene consisting of 3521 bases is presented in SEQ ID No.: 1 of the Sequence Listing. Sequencing was done at least three times.

A site difficult to sequence was subcloned to sequence starting from both terminals. The base sequence from 2880 to 3521 shown in SEQ ID No.: 1 is already reported (Biochem. Biophys. Res. Comm. 204, 646-652, 1994).

Example 3 : Construction of a recombinant vector containing the human MP52 gene promoter and a luciferase reporter gene

The restriction enzyme map of the 5' upstream region 3.5 kb of human MP52 gene is shown in Fig. 1. An *Apa*LI-*Bgl*I fragment (0.6 kb) of the 5' upstream region of human MP52 gene was purified and the *Apa*LI end was blunted. Then, this fragment was treated with *Hin*dIII and *Bgl*II and inserted into a pGL3-basic vector of which *Hin*dIII end was blunted. The vector obtained was treated with *Bgl*II and *Kpn*I restriction enzymes to ligate to the *Bgl*II-*Kpn*I fragment (derived from pBluescript vector) (2.7 kb) of the 5' upstream region of the human MP52 gene. The recombinant expression vector pMP52L (8085 bp) finally obtained contains 3.3 kb of 5' upstream region of human MP52 gene. Fig. 2 shows the constructed recombinant expression vector.

Example 4 : Measurement of activity of the human MP52 promoter (introduction of the recombinant expression vector into human cells and transient expression)

In order to transiently express the recombinant expression vector pMP52L of human MP52 promoter, said vector was mixed with a vector pRL-SV40 (provided from of Pro Mega Ltd.) containing a sea pansy luciferase gene as an internal control for measurement of introducing efficiency of a gene in an equal quantity. In addition, cationic liposome lipofectamine (provided from Lifetech Oriental Co.) was mixed with said DNA solution to add to human osteosarcoma cells; HOS, MG63, or SaOS-2, for transfection. The fire fly luciferase activity and the sea pansy luciferase activity were measured by Pikka Gene Dual Kit (provided from Toyo Ink Co.). The result is shown in Fig. 3. The promoter activity was expressed as a ratio of the fire fly luciferase activity to the sea pansy luciferase activity. From the result, it has been known that the DNA of SEQ ID No.: 1 of the Sequence Listing has a promoter activity.

Example 5 : Introduction of the recombinant expression vector into human cells and stabilized expression

In order to express stably the recombinant expression vector pMP52L of human MP52 promoter, said vector was mixed with a plasmid pPUR containing a gene resistant to puromycin in a proportion of 10 : 1, mixed with cationic liposome lipofectamine (provided from Lifetech Oriental Co.) and added to SaOS-2 cells for transfection. The cells into which the target gene has been introduced were selected from a culture medium containing puromycin (provided from Sigma Ltd.).

Example 6 : Screening of active low molecular weight compound

The cells selected were inoculated in a 96-well plate, treated with substances of various chemical compound libraries for 1 - 3 days, dissolved with cytolytic agent (provided from Pro Mega Ltd.), and measured for enzyme activity by employing a luciferase assay kit (provided from Pro Mega Ltd.). By such steps, various substances enhancing or inhibiting the expression of human MP52 can be surveyed.

Sequence Listing Free Text

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<223> Human MP52 5' upstream gene sequence ; the initiating codon ATG is located at the end of the sequence.

<210> 2
<223> Sense PCR primer OD corresponding to human MP52 cDNA sequence

<210> 3
<223> Reverse PCR primer OID corresponding to human MP52 cDNA sequence

What is claimed is:

1. A DNA whose nucleotide sequence is represented by the base sequence from No. 1 to No. 3521 in SEQ ID NO.: 1 of the Sequence Listing which encodes a human MP52 gene promoter region, or a fragment thereof.

2. A DNA whose nucleotide sequence is represented by the base sequence from No. 1 to No. 2879 in SEQ ID NO.: 1 of the Sequence Listing which encodes a human MP52 gene promoter region, or the fragment thereof.

3. A method for preparing the DNA shown in SEQ ID NO.: 1 of the Sequence Listing comprising the steps of:

(1) preparation of a cDNA probe by PCR with a degenerate primer,

(2) plaque hybridization screening of a human genomic phage library by using the probe, and

(3) subcloning of a target fragment from an isolated phage clone.

4. A recombinant expression vector characterized by integration of the full length or a part of DNA shown in SEQ ID NO.: 1 of the Sequence Listing into a reporter gene.

5. A method for exploring a useful substance, characterized by using the recombinant expression vector according to claim 4.

6. The method for exploring a useful substance according to claim 5, wherein the useful substance is a bone-related substance.

7. The method for exploring a useful substance according to claim 6, wherein the bone-related substance is an osteogenesis inducing substance.

8. The method for exploring a useful substance according to claim 6, wherein the bone-related substance is an osteogenesis inhibiting substance.

9. The method for exploring a useful substance according to claim 6, wherein the useful substance is a neurogenic substance.

10. The method for exploring a useful substance according to claim 6, wherein the useful substance is an angiogenic

substance.

11. The method for exploring a useful substance according to claim 6, wherein the useful substance is an anti-angiogenic substance.

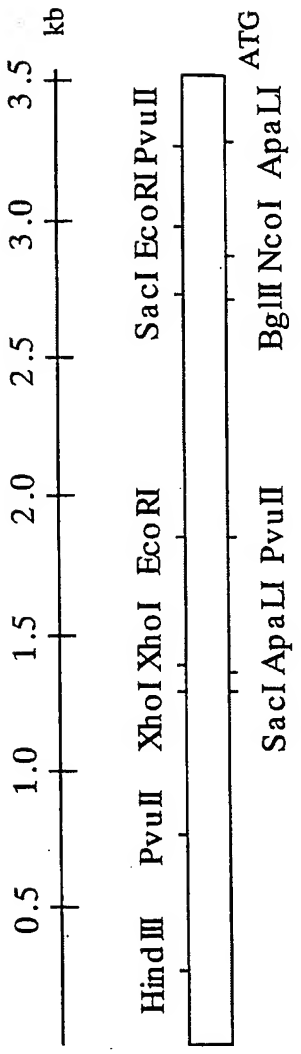
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FIGURE 1

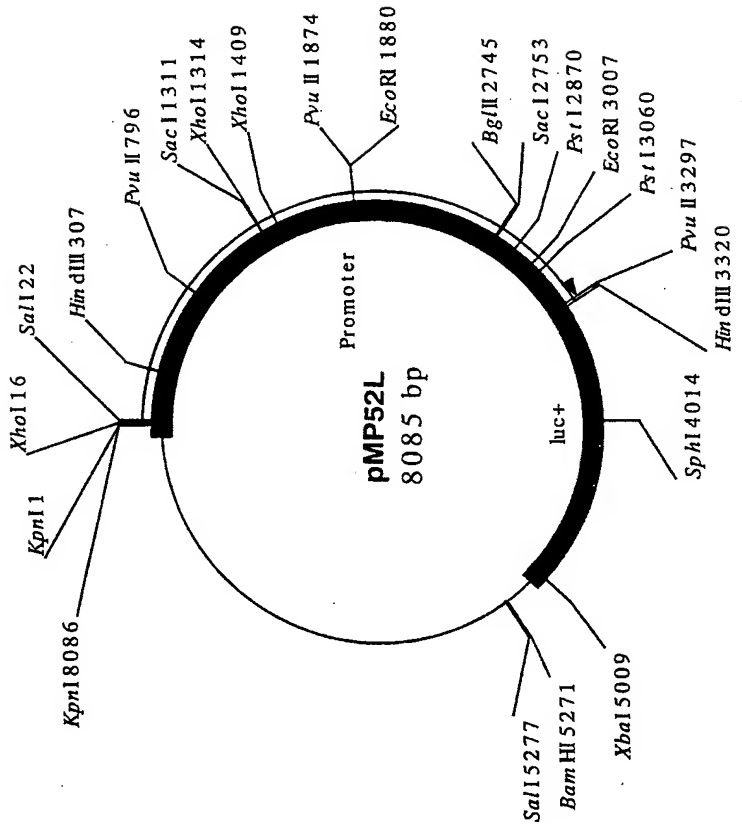

FIGURE 2

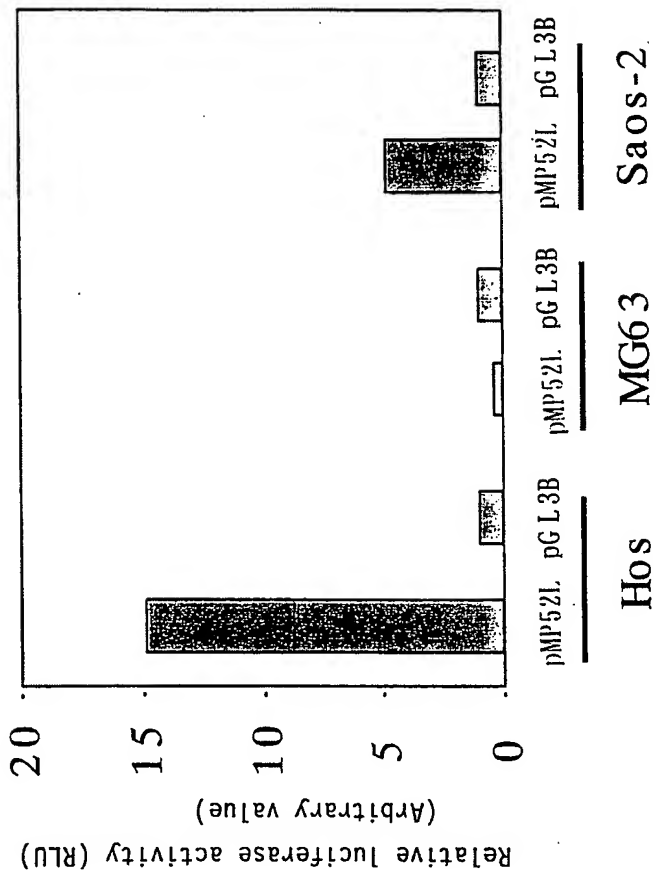

FIGURE 3

SEQUENCE LISTING

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<221> misc_feature

<222> (1)..(29)

<223> Reverse PCR primer OID corresponding to human MP52 cDNA sequence

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INTERNATIONAL SEARCH REPORT

Internat l Application No
PCT/IB 99/01071

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/85 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 September 1999	08/10/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Lonnoy, O

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INTERNATIONAL SEARCH REPORT

Interna: I Application No
PCT/IB 99/01071

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	Seq.Id.No.2	2-11
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